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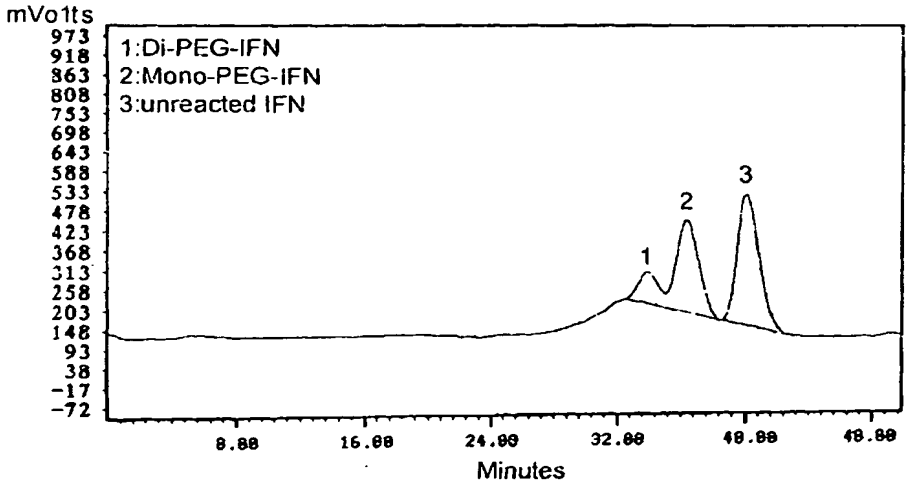
(54) Title: HIGHLY REACTIVE BRANCHED POLYMER AND PROTEINS OR PEPTIDES CONJUGATED WITH THE POLYMER



(57) Abstract: The present invention relates to new biocompatible polymer derivatives, and a protein-polymer or a peptide-polymer which is produced by conjugation of biologically active protein and peptide with the biocompatible polymer derivatives. More particularly, the present invention relates to a highly reactive branched biocompatible polymer derivative containing a long linker between polymer derivatives and protein or peptide molecules, which is minimized in decrease the biological activity of proteins by conjugating the less number of polymer derivatives to the active sites of proteins, improved in water solubility, and protected from being degraded by protease. In hence, the highly reactive branched biocompatible polymer-proteins or peptides conjugates with long linker retain the biological activity in a long period of time and improve a bioavailability of bioactive proteins and peptides.

WO 02/09766 A1

HIGHLY REACTIVE BRANCHED POLYMER AND PROTEINS OR PEPTIDES CONJUGATED WITH THE POLYMER

FIELD OF THE INVENTION

The present invention relates to new biocompatible polymer derivatives, and a protein-polymer or a peptide-polymer which is produced by conjugation of biologically active protein and peptide with the biocompatible polymer derivatives. More particularly, the present invention relates to a highly reactive branched biocompatible polymer derivatives containing a long linker between polymer derivatives and protein or peptide molecules, which is minimized in decrease the biological activity of proteins by conjugating the less number of polymer derivatives to the active sites of proteins, improved in water solubility, and protected from being degraded by protease. In hence, the highly reactive branched biocompatible polymer-proteins or peptides conjugates with long linker retain the biological activity in a long period of time and improve a bioavailability of bioactive proteins and peptides.

BACKGROUND ART OF THE INVENTION

In general, various proteins and peptides such as hormones and cytokines play important roles in the body.

With a recent great advance in genetic engineering, various proteins have been manufactured in a mass scale and used as therapeutic drugs.

Use of these proteins and peptides as medicines, however, suffers from many problems. First, peptides or proteins are very low in body absorption efficiency because they are easily hydrolyzed or degraded by enzymes within a short period of time after being taken into the body. Further, when such proteins and peptides drugs are repetitively administered, immune reactions are frequently induced to produce antibodies which may cause very serious hypersensitivity as to menace the life of the patients, acting as a neutralizing role against the physiological activity of drugs. In addition, the clearance attributable to the reticuloendothelial system (RES) is increased. Therefore, most protein and peptide drugs have been administered by injection, thus far. The administration by injection, however, gives the patients pain accompanied dangers. Particularly, patients who need to be treated for a long period of time may not be able to treat themselves by injection. Thus, there remains a need to develop more stable therapeutic protein or peptides drugs.

Conjugation of pharmaceutically active proteins or peptides to synthetic macromolecules may afford great

advantages when they are applied *in vivo* and *in vitro*. When being covalently bonded to macromolecules, physiologically active molecules may be changed in surface properties and solubility. Further, the presence of macromolecules may make the conjugated proteins and peptides more stable *in vivo* as well as reduce the clearance attributed to the intestinal system, the kidney, the spleen, and/or the liver. Hence, conjunction of polymers to proteins or peptides can bring about a great improvement in the stability of proteins and peptides in solutions and effectively protect the intrinsic surface properties of peptides to prevent non-specific protein adsorption.

U. S. Patent No. 4,179,337 discloses conjugates between peptides or polypeptides and polyethylene glycol (hereinafter, referred to as "PEG") with a molecular weight of 500~20,000 or water-soluble polymers, which are reduced in antigenicity and immunogenicity while maintaining the biological activity of the proteins and polypeptides. It is described in U. S. Patent No. 4,301,144 that hemoglobin is increased in oxygen molecule-carrying potential when being associated with PEG or water-soluble polymers.

Various proteins are reported to show extended half-life spans and reduced immunogenicity in plasma when being conjugated with PEG (Abuchowski et al., *Cancer Biochem.*

Biophys., 7, 175-186, **1984**). Uricase-PEG conjugates are demonstrated to be increased *in vivo* half-life span and show the reduced side-effect during the metabolism of uric acid (Davis et al., *Lancet*, 2, 281-283, **1981**).

As apparent from the preceding patents the conjugation of PEG allows biologically active proteins and peptides to increase *in vivo* half-life span and solubility and to reduce the immune reactions.

The conjugation of PEG to proteins or peptides is achieved by reacting activated PEG to amino residues of proteins or peptides, lysine residues and N-termini. As for PEG activation, one of the hydroxyl groups of PEG is substituted with a methyl ether group while the other hydroxy group is bonded to an electrophilic functional group (Abuchowski, A. and Davis, F. F. (1981), in Enzymes as Drugs (Holsenberg, J. and Roberts, *J., eds.*)). Examples of activated polymers include PEG-N-hydroxysuccinimide active esters, which contain amide bonds, PEG-epoxides and PEG-tresylate, which contain alkyl bonds, PEG-carbonyl imidazole and PEG-nitrophenyl carbonates, which contain urethane bonds, and PEG-aldehyde, which contains a Schiff's base at the N-terminus.

On a polypeptide sequence, lysine residues are randomly located, so that PEG is non-specifically bonded to

the proteins or polypeptides. In order to obtain uniformed PEG-peptide conjugates, there have been made attempts of bonding PEG to targeted sites such as cystein residues, oligo sugars, hydroxyl groups, and arginine groups.

Examples of PEG derivatives being able to specifically react to cystein groups of polypeptides include PEG-vinyl sulfone, PEG-iodoacetamide, PEG-maleimide, and PEG-orthopyridyl disulfide. PEG-vinyl sulfone is the best from the view of the stability in water solutions while PEG-orthopyridyl disulfide can be reversibly degraded *in vivo* because of the presence of disulfide bonds. Peptides taking advantage of these derivatives can be exemplified by Interleukin-3 and Interleukin-2.

PEG derivatives reacted specifically to oligo sugars of polypeptides may be exemplified by PEG-hydrazides, which is able to react with aldehyde containing compounds to form relatively stable hydrazone bonds. Advantage is taken of the specific bonding of PEG-hydrazides to sugar moieties or glycoproteins.

PEG-isocyanates react specifically with hydroxy groups of polypeptides. In order to conjugate PEG to arginine residues of polypeptides, PEG derivatives containing phenylglyoxal which is highly reactive to the guanidino group have been used.

General structure of polyethylene is a linear having molecular weight of between 1,000 Da and 25,000 Da. However, there is a barrier to conjugate a number of linear polymers to proteins or peptides with retaining the biological activity because the active sites in proteins or peptides are limited. Particularly, polymer conjugation to low molecular weight of proteins or peptides results in a significant decrease of biological activity by steric hindrance because a number of active sites are relatively low. Thus, there have been many attempts to conjugate large polymers to proteins or peptide with retaining the biological activity. First, the conjugation of linear polymers with a molecular weight of 20,000 and higher has been attempted and resulted in the extended the circulating half-life compared to polymers with a molecular weight of less than 20,000 Da. However, the yield of this conjugation was found to be very low and considered not to be economic.

To overcome the problem of linear polymer conjugating proteins or peptides as mentioned above, the use of branched PEG for the conjugation has been attempted by Wana (Wana, H. et al., 'Antitumor enzymes: polyethylene glycol-modified asparaginase', *Ann. N. Y. Acad. Sci.* 613, 95-108, **1990**). It was reported that the proteins or peptides were conjugated to the branched mPEG derivatives by

trichlorotriazine. However, mPEG-disubstituted chlorotriazine and the process of preparation thereof, are still present severe limitations because coupling to protein is highly nonselective. Several types of amino acids other than lysine are attached and many proteins are inactivated.

Yamasaki (Yamasaki, N. et al., *Agric. Biol. Chem.*, 52, 2125-2127, **1988**) has inserted norleucine in the process to synthesize the branched mPEG in order to analyze easily. This method provides the advantage to calculate the ratio between polymers and protein molecules by determining the number of norleucine in amino acid analysis.

Also, U.S Patent No. 5,932,462 and No. 5,643,575 disclosed a branched or multi-armed aliphatic polymer derivative that is monofuntional, hydrolytically stable. However, these branched polymers with short length of linker between polymer and protein cause the steric hindrance and in hence reduce the reactivity and yield of product.

To overcome the foregoing problems, we, the inventors of the present invention, have developed branched polymer with long length of linker to conjugate to proteins. The present invention has confirmed that the steric hindrance has been decreased and the reduction of biological activity

has been minimized by being protected from degradation by proteases.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a branched biocompatible polymer with long length of linker to conjugate with protein or peptide.

Another object of the invention is also to provide the stable and water soluble protein-polymer or peptide-polymer conjugates that reduce the steric hindrance in active sites of proteins and retain the biological activity.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a size exclusion chromatography (SEC) of intact interferon (IFN), which is not conjugated with polymer derivatives.

Fig. 2 represents a graph of SEC which IFN reacted with activated Di-PEG5000;

where 1: PEG_2-IFN, 2: PEG_1-IFN, 3: unreacted IFN.

Fig. 3 shows a graph of SEC of IFN reacted with activated Di-PEG20000;

where 1: PEG_1-IFN, 2: unreacted IFN.

Fig 4 represents a graph of SEC which IFN reacted with activated Tri-PEG5000;

where 1: PEG_2-IFN, 2: PEG_1-IFN, 3: unreacted IFN.

Fig. 5 represents a graph of SEC which IFN reacted with activated Tri-PEG20000;

where 1: PEG_2-IFN, 2: PEG_1-IFN, 3: unreacted IFN.

DETAILED DESCRIPTION OF THE INVENTION

In order to accomplish the aforementioned goal, the present invention provides a branched biocompatible polymer with long length of linker to conjugate with protein or peptide.

Further, the present invention also provides the stable and water soluble protein-polymer or peptide-polymer conjugates that reduce the steric hindrance in active sites of proteins and retain the biological activity.

Further features of the present invention will appear hereinafter.

The branched biocompatible polymer according to the present invention is represented by the following formula 1:

FORMULA 1

$$(P\text{-}OCH_2CO\text{-}NH\text{-}CHR\text{-}CO\text{-})_n\text{-}L\text{-}Q_k\text{-}A$$

Wherein,

P and Q is the same or different biocompatible polymer,

R is H or alkyl,

L is aliphatic linking moiety covalently linked to each P and Q,

A is activating functional group,

n is an integer between 2 and 3,

k is an integer between 0 and 1.

The biocompatible polymer derivatives in the present invention are the activated branched polymers prepared by bonding one or more biocompatible polymers. In this regard, the bond between the polymers and protein or peptide may be a covalent bond or a non-covalent bond such as a lipophilic bond or a hydrophobic bond. In preparing highly reactive branched polymers, the biocompatible polymer has been activated and reacted to each other to provide a branched polymer derivatives (Di-polymer derivatives). A branched biocompatible polymer derivatives (Tri-polymer derivatives) containing long length of linker at branched point to conjugate with protein and peptide can be provided as a preferred example of the present invention.

The term "biocompatible polymers" as used herein

means naturally occurring or synthetic compounds which are dissolved in water. By way of example, not limitation, the biocompatible polymers(represented by P and Q) include polyethylene glycol (PEG), polypropylene glycol (PPG), polyoxyethylene (POE), polytrimethylene glycol, polylactic acid and its derivatives, polyacrylic acid and their derivatives, polyamino acid, polyvinyl alcohol, polyurethane, polyphosphazene, poly(L-lysine), polyalkylene oxide (PAO), and water soluble polymers such as polysaccharide, dextran, and non immunogenic polymers such as polyvinyl alcohol and polyacryl amide.

Available in the present invention are the polymers used to synthesize the branched polymer derivatives ranging in molecular weight from about 200 to 100,000 and preferably from 1,000 to 40,000.

The liker of branched polymer derivatives to conjugate with protein or peptide in the present invention is a long length of activated biocompatible polymers and the polymers ranging in the molecular weight preferably from 2,000 to 20,000 are available.

A method of branched polymer in the present invention can be proceeded to activate polymers by inserting a linker(represented by L) containing aliphatic amino acid linking moiety into functional group having reactivity. The

functional groups(represented by A) of the present polymer derivatives can be N-hydroxysuccinimide ester (hereinafter, referred to as "NHS"), hydrazine hydrate (hereinafter referred to as "NH_2NH_2"), carbonyl imidazole, nitrophenyl, isocyanate, sulfonyl chloride, aldehyde, glyoxal, epoxide, carbonate, cyanuric halide, dithiocarbonate, tosylate, and maleimide and preferably NHS or NH_2NH_2.

A method of polymer activation comprises the following steps of:

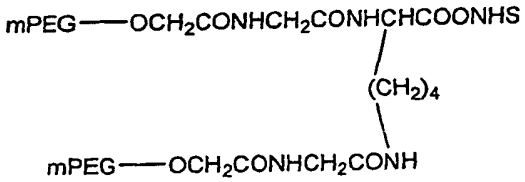
(a) preparing the polymer into polyalkylene oxide (hereinafter, referred to as "PAO") such as monomethoxy-poly(ethylene glycol) (hereinafter referred to as "mPEG"); and,

(b) changing the other part of PAO into a reaction group having reactivity.

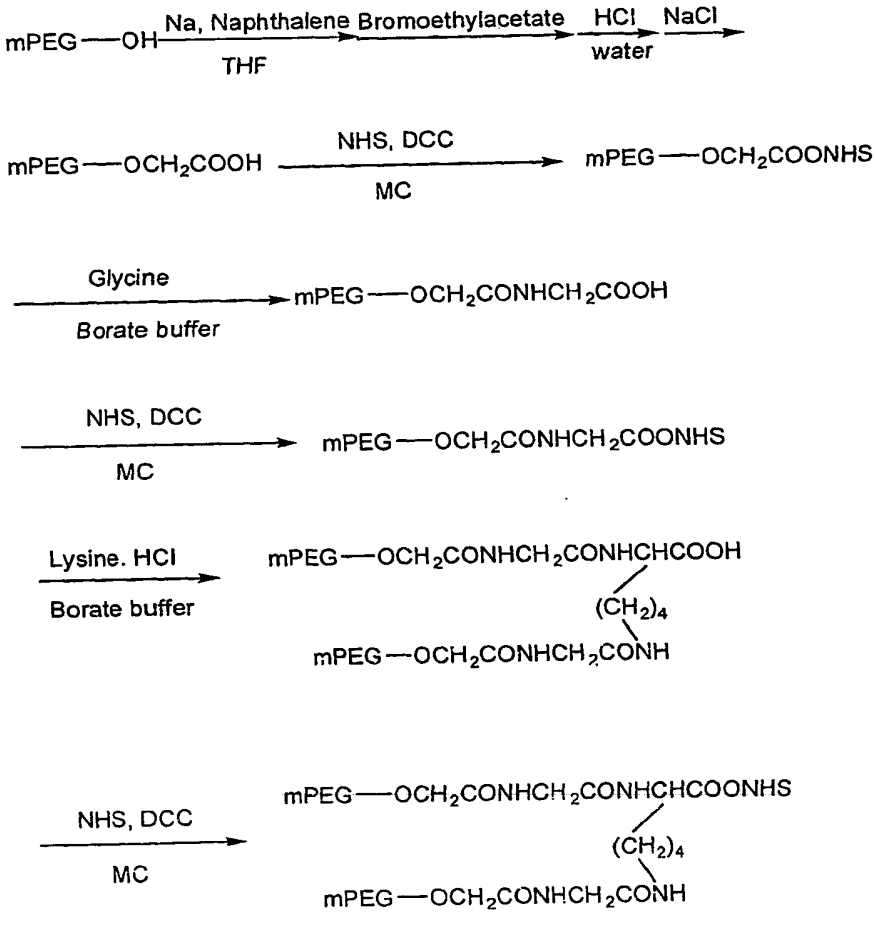
Particularly a method for activating the biocompatible polymer by NHS is shown the following Scheme 1 and Scheme 2.

Scheme 1 illustrates the procedure for preparation of activated Di-polymer derivatives, represented by the following formula 2, containing activated branched polymer.

FORMULA 2



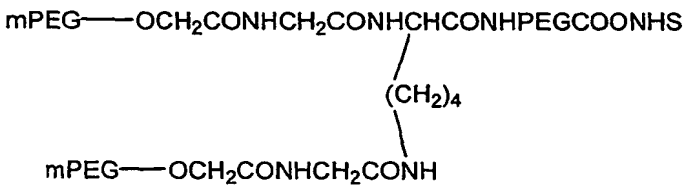
SCHEME 1



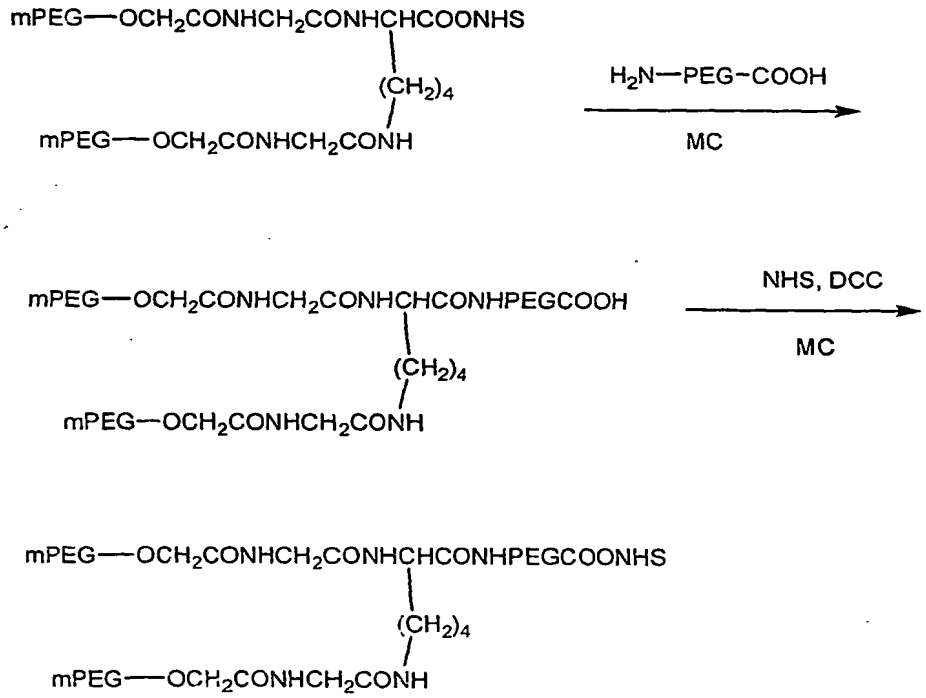
Scheme 2 shows the method for preparation of activated Tri-polymer derivative, represented by the following formula 3, that was prepared by reacting an

activated Di-polymer derivative with activated polymer containing a long length of linker to conjugate to proteins or peptides.

FORMULA 3



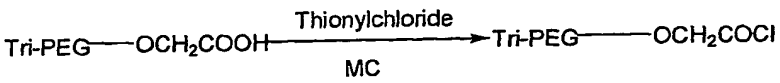
SCHEME 2

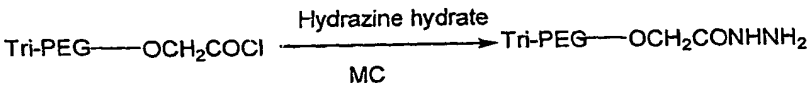


As reacting groups of activated branched polymer derivatives for conjugating to proteins or peptides, NH_2NH_2, carbonyl imidazol, nitrophenyl, isocyanate, sulfonyl

chloride, aldehyde, glyoxal, epoxide, carbonate, cyanuric halide, dithiocarbonate, tosylate and maleimide can be used as well as NHS, where the use of NH_2NH_2 was shown in Scheme 3.

SCHEME 3





The present invention also provides protein-polymer or peptide-polymer conjugates with activated branched polymer derivatives synthesized in this invention. .

As described above, the present invention provides highly reactive protein or peptide-polymer conjugates prepared by reacting activated branched polymer with biologically active protein or peptide. In this regard, the bond between the protein or peptide and the polymer derivatives may be a covalent bond or a non covalent bond such as a lipophilic bond or a hydrophobic bond.

The activated branched polymer forms the protein or peptide polymer conjugates by reacting with ε-amine group of lysine. Besides the amine group of lysine, carboxyl group, activated carbonyl group, oxidized sugar and

mercapto group in the protein can be used as a conjugated moiety to the activated branched polymer.

The conjugation of biologically active protein or peptide with one or more activated branched polymers can be prepared by chemical reaction and the temperature of conjugation reaction is in the range of 0 to 40 ^{0}C and preferably in the range of 4 to 30 ^{0}C. In the range of 4 to 9 for the reaction pH and 5 minutes to 10 hours for the reaction time are preferable in this preparation. Also the molar ratio of protein or peptide polymer conjugates is in the range of 1:1 to 1:100 and preferably in the range of about 1:1 to 1:20.

The protein or peptide of the present invention is not limited to the specific therapeutic agents but applied to the all substances having biological activity, particularly, it is desirable to use alpha -, beta-, gamma-interferon (hereinafter referred to as IFN), asparaginase, arginase, arginine deiminase, adenosine deaminase, superoxide dismutase, endotoxinase, catalase, chymotrypsin, lipase, uricase, adenosine diphosphatase, tyrosinase, glucose oxidase, glucosidase, galactosidase, glucouronidase, hemoglobin, blood factors (VII, VIII and IX), immunoglobulins, cytokines such as interleukins, G-CSF, GM-

CSF, PDGF, lectins, ricins, TNF, TGFs, epidermal growth factor (hereinafter referred to as EGF), human growth hormone (hereinafter referred to as hGH), calcitonin, PTH, insulin, enkephalin, GHRP, LHRH and derivatives, calcitonin gene related peptide, thyroid stimulating hormone and thymic humoral factor.

The activated branched polymer derivatives in the present invention show the high reactivity to conjugate with proteins or peptides. Particularly the reactivity of activated biocompatible polymer to proteins or peptides in the case of Tri-polymer derivatives was confirmed to be very high compared to Di-polymer derivatives (refer to Fig. 2-5 and Table). Therefore, it was found that the long length of linker of Tri-polymer enhanced the reactivity with proteins or peptides as described above.

The purification of protein or peptide-polymer conjugates is performed in buffer solution in the pH range of 7 to 9 and preferably 7.5 to 8.5. The buffer solutions used in the purification step can be KCl, NaCl, Tris-HCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, $NaHCO_3$, $NaBO_4$, $(NH_4)_2CO_3$, glycine-NaOH and preferably Tris-HCl and phosphate buffer solutions. In addition, ion exchange resins used in the

present invention can be Q-HD (Biosepra, USA), QA-Trisacryl and QMA-Spherosil (Sepracore, USA), TMAE650M (EM separation, USA), Mono-Q and Q-Sepharose (Pharmacia, Sweden).

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples. However, it will be appreciated that those skills in the art, on consideration of this disclosure, would make modifications and improvements within the spirit and scope of the present invention.

1. Preparation of activated PEG derivatives

<Example 1> Preparation of activated mPEG-$OCH_2CONHCH_2COONHS$ (5000)

<1-1> Preparation of mPEG-OCH_2COOH (5000)

Mono methoxy-poly(ethylene glycol) was prepared from PEG (MW 5000) so that one hydoxyl group of PEG was protected. 10 g of mPEG-OH(5000) (2 mmole) was dissolved in THF under nitrogen gas, added to sodium and naphthalene solution, and stirred for 3 hours at room temperature. 1 g of bromoethylacetate (6 mmole) was added drop wise at room temperature with stirring. After 15 hours, the product was precipitated in ether on ice bath. The crude solid was filtered, washed with ether, collected and dried under

vacuum. 15.5 g of crude solid was obtained.

The crude solid prepared as described above was dissolved in d-H_2O and the pH was adjusted to 11 with 1 N NaOH. After stirring for 24 hours, it was cooled to room temperature and the pH was adjusted to 3 with 1 N HCl prior to dryness. The solid was then dissolved in methylene chloride (hereinafter, referred to as "MC"), left at room temperature for 1 hour, and filtered using the celite prior to dryness. The crude solid was recrystallized in isopropyl alcohol (hereinafter, referred to as "IPA") on ice bath. The pale brown solid was then obtained, filtered, and rinsed with ether prior to dryness under vacuum. The yield was calculated to be 100 % (10.3 g).

<1-2> Preparation of mPEG-$OCH_2COONHS$ (5000)

3 g of mPEG-OCH_2COOH (5000) (0.6 mmole) prepared in the Example <1-1> was dissolved in MC and added to 0.2 g of NHS (1.8 mmole) and 0.3 g of N,N'-dicyclohexyl carbodiimide (1.8 mmole) (hereinafter, referred to as "DCC") with stirring. The reaction was carried out at 30 ^{0}C for 18 hours with stirring and cooled to room temperature followed by filtration using celite and charcoal consequently prior to dryness. The solid product was crystallized in IPA on ice bath, filtered, rinsed with ether, and dried under

vacuum. 2.81 g of mPEG-$OCH_2COONHS$ was obtained (yield: 91 %).

<1-3> Preparation of mPEG-$OCH_2CONHCH_2COOH$ (5000)

To 0.06 g of glycine (0.8 mmole) in 0.1 M borate buffer solution, pH 8.5, was added 0.5 g of mPEG-$OCH_2COONHS$ (5000) (0. 1 mmole) drop by drop. After reacting for 36 hours at room temperature, d-H_2O was added and pH was adjusted to 3 by adding oxalic acid. The reaction mixture was extracted in MC three times and the separated layer was dried after addition of Na_2SO_4. The solid product was crystallized in IPA, washed with ether after filtration, and dried under vacuum. 0.5 g of mPEG-$OCH_2CONHCH_2COOH$ (5000) was obtained. The yield was 98 %.

<1-4> Preparation of mPEG-$OCH_2CONHCH_2COONHS$ (5000)

0.5 g of mPEG-OCH_2COOH (5000) (0.1 mmole) prepared in the Example <1-3> was dissolved in MC and added to 0.034 g of NHS (0.3 mmole) and 0.062 g of DCC (0.3 mmole) with stirring. The reaction was carried out at 30 ^0C for 24 hours with stirring and cooled to room temperature followed by filtration using celite and charcoal consequently prior to dryness. The solid product was crystallized in IPA on ice bath, filtered, rinsed with ether, and dried under

vacuum. 0.43 g of mPEG-$OCH_2CONHCH_2COONHS$ (5000) was obtained (yield: 83 %).

<Example 2> Preparation of activated mPEG-$OCH_2CONHCH_2COONHS$ (20000)

<2-1> Preparation of mPEG-OCH_2COOH (20000)

5 g of mPEG-OH(20000) (0.25 mmole) was prepared as the same method described in <Example 1-1> and 5 g of solid product, mPEG-OCH_2COOH (20000), was obtained. The yield was calculated to be 100 %.

<2-2> Preparation of mPEG-$OCH_2COONHS$ (20000)

3 g of mPEG-OCH_2COOH (20000) (0.15 mmole) was prepared as the same method described in <Example 1-2> and 2.2 g of solid product, mPEG-$OCH_2COONHS$ (20000), was obtained. The yield was calculated to be 73 %.

<2-3> Preparation of mPEG-$OCH_2CONHCH_2COOH$ (20000)

0.5 g of mPEG-$OCH_2COONHS$ (20000) (0.025 mmole) was prepared as the same method described in <Example 1-3> and 0.5 g of solid product, mPEG-$OCH_2CONHCH_2COOH$ (20000), was obtained. The yield was calculated to be 100 %.

<2-4> Preparation of mPEG-$OCH_2CONHCH_2COONHS$ (20000)

0.5 g of mPEG-$OCH_2CONHCH_2COOH$(20000) (0.025 mmole) was prepared as the same method described in <Example 1-4> and 0.45 g of solid product, mPEG-$OCH_2CONHCH_2COONHS$ (20000), was obtained. The yield was calculated to be 90 %.

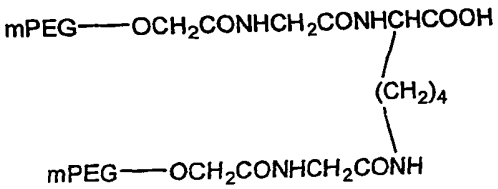
2. Preparation of activated branched Di-PEG and Tri-PEG derivatives

<Example 3> Preparation of activated branched Di-PEG-NHS(5000)

<3-1> Preparation of Di-PEG-COOH(5000)

0.4 g of mPEG-$OCH_2CONHCH_2COONHS$ (5000) (0.076 mmole) was added to 0.08 g of lysine-HCl (0.042 mmole) in 0.1 M borate buffer solution, pH 8.5. After completion of reaction for 48 hours at room temperature, d-H_2O was added and the pH of the solution was adjusted to 3 with oxalic acid. The reaction mixture was extracted in MC three times and the separated layer was dried after adding Na_2SO_4. The solid product was crystallized in IPA, washed with ether after filtration, and dried under vacuum. 0.33 g (yield of 84 %) of white solid product, Di-PEG-COOH(5000) was obtained. The resulting solid product has the formula as illustrated in formula 4.

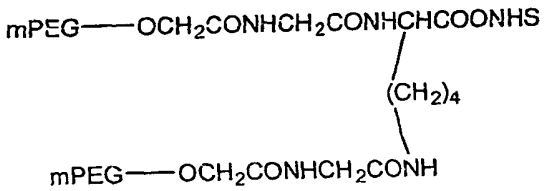
FORMULA 4



<3-2> Preparation of Di-PEG-NHS(5000)

0.3 g of Di-PEG-COOH(5000) (0.029 mmole) prepared in the example 3-1, was dissolved in MC and added to 0.01 g of NHS (0.087 mmole) and 0.018 g of DCC (0.087 mmole) with stirring. Di-PEG-NHS(5000)was then prepared as the same method described in example 1-4 and 0.25 g of solid product (yield of 82 %), Di-PEG-NHS(5000), was obtained. The resulting solid product has the formula as illustrated in formula 2.

FORMULA 2



<Example 4> Preparation of activated branched Di-PEG-NHS(20000)

<4-1> Preparation of Di-PEG-COOH(20000)

0.4 g of mPEG-$OCH_2CONHCH_2COONHS$ (20000) (0.02 mmole)

was used to obtain 0.35 g of white solid product, Di-PEG-COOH(20000) (yield of 87 %) by following the same procedure as described in <Example3-1>. The resulting product has a formula as illustrated in Formula 1 except that the molecular weight of PEG in this formula is 20,000.

<4-2> Preparation of Di-PEG-NHS(20000)

0.3 g of Di-PEG-COOH(20000) (0.025 mmole) was used to obtain 0.25 g of white solid product, Di-PEG-COOH(20000) (yield of 83 %) by following the same procedure as described in <Example 3-2>. The resulting product has a formula as illustrated in Formula 2 except that the molecular weight of PEG in this formula is 20,000.

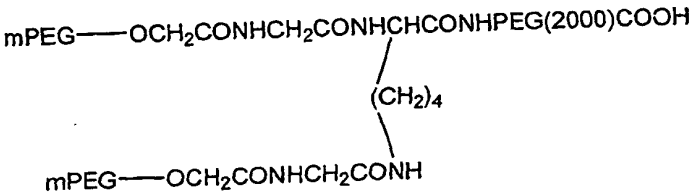
<Example 5> Preparation of activated branched polymer derivatives with long length of linker, Tri-PEG-NHS (5000)

<5-1> Preparation of Tri-PEG-COOH (5000)

0.1 g of Di-PEG-COONHS(5000) (0.0096 mmole) prepared in the <Example 3> was dissolved in MC and was added NH_2PEG-COOH(2000) (0.038 g, 0.0192 mmole) at room temperature with stirring. After completion of reaction for 48 hours at 40 ^{0}C, the reaction mixture was filtered using celite and evaporated to dryness. The solid product was crystallized in IPA, washed with ether after filtration,

and dried under vacuum. 0.12 g (yield of 92 %) of white solid product, Tri-PEG-COOH(5000) was obtained. The resulting solid product has the formula as illustrated in formula 5.

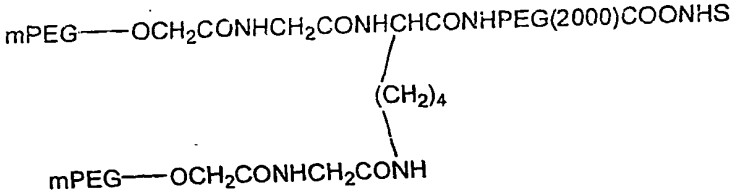
FORMULA 5



<4-2> Preparation of Tri-PEG-NHS (5000)

0.1 g of Tri-PEG-COOH(5000) (0.007 mmole) prepared in <Example 4-1> was reacted with 0.0024 g of NHS (0.021 mmole) and 0.0043 g of DCC (0.021 mmole) in MC as the same method described in <Example 3-2>, and 0.1 g of solid product (yield of 99 %), Tri-PEG-NHS(5000), was then obtained. The resulting solid product has the formula as illustrated in formula 6.

FORMULA 6



<Example 6> Preparation of activated branched polymer

derivatives with long length of linker, Tri-PEG-NHS (20000)

<6-1> Preparation of Tri-PEG-COOH (20000)

0.1 g of Di-PEG-COONHS(20000) (0.00247 mmole) prepared in <Example 4> was reacted as the same method described in <Example 5-1>, and 0.107 g of solid product with a yield of 98 % was obtained. The resulting product, Tri-PEG-COOH, has a formula as illustrated in formula 5 except that the molecular weight of PEG in this formula is 20,000.

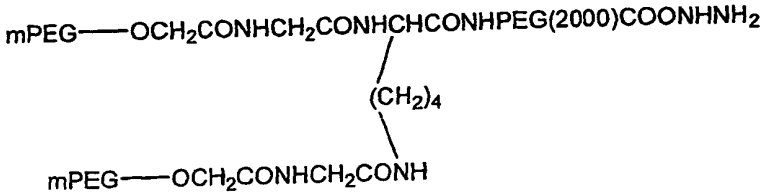
<6-2> Preparation of Tri-PEG-NHS (20000)

0.08 g of Tri-PEG-COOH(20000) (0.0018 mmole) prepared in <Example 6-1> was reacted as the same method described in <Example 5-2> and 0.08 g of solid product (yield of 99 %), Tri-PEG-NHS(20000), was then obtained. The resulting solid product has the formula as illustrated in formula 6 except that the molecular weight of PEG in this formula is 20,000.

<Example 7> Preparation of Tri-PEG-$NHNH_2$ (5000)

The product illustrated in Formula 7 was prepared as following procedures in <Example 6-1> and <Example 6-2>.

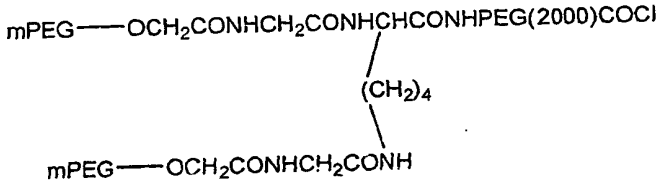
FORMULA 7



<7-1> Preparation of PEG derivative, (Tri-PEG-COCl)

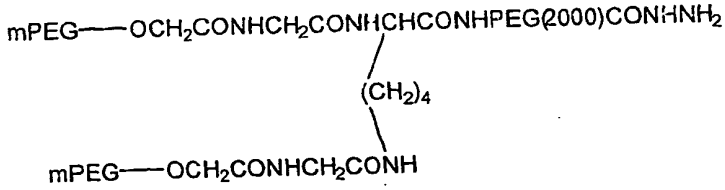
1 g of Tri-PEG-COOH (5000) (0.083 mmole) prepared in <Example 5-1> was reacted with 0.05 g of $SOCl_2$ (0.4 mmole) in MC, the reaction mixture was refluxed for 3 hours with heating, and cooled to room temperature prior to evaporation. 1 g of brown oil (yield: 98 %) was obtained, which need to be used immediately due to instability.

FORMULA 8



<7-2> Preparation of PEG derivative, ($Tri-PEG-CONHNH_2$)

FORMULA 9



1.1 mmole of Tri-PEG-COCl (5000) prepared in the step

1 in MC, was reacted with NH_2NH_2 and 10 ml of d-H_2O for 3 hours at room temperature with stirring and the reaction mixture was purified on silica column before evaporation and dried under vacuum. As a result, 1 mmole of yellowish oil (yield of 92 %) was obtained in the formula of Formula 9.

<Example 8> Preparation of Tri-PEG-$NHNH_2$ (20000)

<8-1> Preparation of PEG derivative in Formula 8

Tri-PEG-COCl (20000) can be prepared from Tri-PEG-COOH (20000) as described in <Example 7-1>. The structure of the resulting product is illustrated in formula 9, except that the molecular weight of PEG in this formula is 20,000.

<8-2> Preparation of PEG derivative in Formula 7

1.1 mmole of Tri-PEG-COCl (20000) prepared in <Example 8-1> was reacted as the same method described in <Example 7-2> to obtain the solid product which has the formula as illustrated in Formula 9 except that the molecular weight of PEG in this formula is 20,000.

3. Preparation of activated PEG-Interferon conjugates

<Example 9> Preparation of Di-PEG(5000)-IFN

3 mg of succinic N-hydroxysuccinimidyl (hereinafter referred to as "SS") Di-PEG (5000) was added to 3 mg of IFN in 0.1 M phosphate buffer solution, pH 7.0 and stirred for 30 minutes at ambient temperature. The reaction was stopped with 0.1 M glycine and the excess reagents were removed by using centricon-30 (Amicon, USA).

<Example 10> Preparation of Di-PEG(20000)-IFN

12 mg of SS-Di-PEG (20000) was added to 3 mg of IFN in 0.1 M phosphate buffer solution, pH 7.0 and stirred for 30 minutes at ambient temperature. The reaction was stopped with 0.1 M glycine and the excess reagents were removed by using centricon-50 (Amicon, USA).

<Example 11> Preparation of Tri-PEG(5000)-IFN

Tri-PEG(5000)-IFN was prepared as indicated in <Example 9>. Tri-PEG(5000)-NHS prepared as described in Example 5 was used.

<Example 12> Preparation of Tri-PEG(20000)-IFN

Tri-PEG(20000)-IFN was prepared as indicated in <Example 9>. Tri-PEG(20000)-NHS prepared as described in Example 6 was used.

<Example 13> Preparation of Tri-PEG(5000)$NHNH_2$-IFN

10 mg of 1-ethyl 3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (hereinafter referred to as "EDC") was added to 3 mg of IFN in 0.1 M phosphate buffer solution, pH 6.0. 3 mg of Tri-PEG(5000)-$NHNH_2$ prepared in <Example 7> was then reacted with the above reaction mixture for 2 to 24 hours at 4 ^{0}C. The excess reagent and unreacted IFN were removed by using centricon-30 (Amicon, USA).

<Example 14> Preparation of Tri-PEG(20000)$NHNH_2$-IFN

10 mg of EDC was added to 3 mg of IFN in 0.1 M phosphate buffer solution, pH 6.0. 12 mg of Tri-PEG(20000)-$NHNH_2$ prepared in <Example 8> was then reacted with the above reaction mixture for 2 to 24 hours at 4 ^{0}C. The excess reagent and unreacted IFN were removed by using centricon-50 (Amicon, USA).

<Example 15> Preparation of Tri-PEG(5000)-EGF

5 mg of SS-Tri-PEG (5000) prepared in <Example 5> was added to 5 mg of EGF in 0.1 M phosphate buffer solution, pH 7.0 and stirred for 30 minutes at ambient temperature. The reaction was stopped with 0.1 M glycine and the excess reagents were removed by using centricon-30 (Amicon, USA).

The separation of desired product was performed as indicated in the <Example 19>.

<Example 16> Preparation of Tri-PEG(20000)-EGF

All procedures were followed as indicated in <Example 15> except that 20 mg of Tri-PEG(20000) was used instead of Tri-PEG(5000). The separation of desired product was performed as indicated in the <Example 19>.

<Example 17> Preparation of Tri-PEG(5000)-hGH

8 mg of SS-Tri-PEG (5000) prepared in <Example 5> was added to 5 mg of hGH in 0.1 M phosphate buffer solution, pH 7.0 and stirred for 30 minutes at ambient temperature. The reaction was stopped with 0.1 M glycine and the excess reagents were removed by using centricon-30 (Amicon, USA). The separation of desired product was performed as indicated in the <Example 19>.

<Example 18> Preparation of Tri-PEG(20000)-hGH

All procedures were followed as indicated in <Example 17> except that 25 mg of Tri-PEG(20000) was used instead of Tri-PEG(5000). The separation of desired product was performed as indicated in the <Example 19>.

<Example 19> Separation of PEG_1(5000 or 20000)-IFN

PEG(5000)-IFN and PEG(20000)-IFN prepared in <Example 9> and <Example 12> were dialyzed to 10 mM Tris buffer solution, pH 8.0. by using centricon-30 or centricon-50, respectively. The PEG_1-IFN that only one PEG attached to one IFN molecule was separated onto anion exchange column using Mono-Q resin. The concentration of NaCl from 0 to 300 mM was used for the linear gradient. The PEG_1-IFN separated above was identified by MALDI-TOF mass spectrometer of size exclusion HPLC.

<Experimental example 1> Comparison of reactivity of activated PEG derivatives

To investigation of reactivity of activated PEG derivatives prepared in <Example 1-8> with proteins or peptides, those derivatives were conjugated with PEG as indicated in <Example 9-14>. After separation of PEG_1-IFN as described in <Example 19> and calculation of the amount of PEG_1-IFN by area of peak in HPLC chromatograms (refer to Fig 2-5), the reactivity of each activated PEG derivatives was compared in Table.

In parallel, the reactivity of commercially available branched PEG with a molecular weight of 40,000 was also compared when the reaction was performed under same

condition.

Table 1. Reactivity of activated PEG derivatives

Activated PEG derivatives		PEG_1-INF produced (%)	Unreacted INF (%)
<Example 1>	mPEG-$OCH_2CONHCH_2COONHS$(5000)	45	35
<Example 2>	mPEG-$OCH_2CONHCH_2COONHS$(20000)	22	57
<Example 3>	Di-PEG-NHS(5000)	23	65
<Example 4>	Di-PEG-NHS(20000)	18	80
<Example 5>	Tri-PEG-NHS(5000)	45	51
<Example 6>	Tri-PEG-NHS(20000)	43	50
<Example 7>	Tri-PEG-$NHNH_2$(5000)	35	30
<Example 8>	Tri-PEG-$NHNH_2$(20000)	30	40
Shearwater		23	63

As a result, Both Di-PEG derivatives and Tri-PEG derivatives showed the reactivity to IFN and particularly Tri-PEG derivatives of the present invention was found to be highly reactive to conjugate with IFN.

INDUSTRIAL APPLICABILITY

The above-mentioned, the biocompatible polymer derivative and protein-polymer or peptide-polymer of he present invention, which are produced by conjugation of biologically active protein and peptide with biocompatible polymer derivatives, are prepared, such that they shows high yield while maintains a biological activity, minimizes activity-decreasing of drug, and increases stability with

inhibiting of decomposition from internal enzyme. Therefore, the highly reactive branched biocompatible polymer-proteins or peptides conjugates according to the present invention, may be effectively used for decreasing of side effects in accordance with over drug abuse, with minimizing the number of administration.

WHAT IS CLAIMED IS;

1. Activated branched biocompatible polymer derivatives comprising a long length of polymer linker with functional group to conjugate with biologically active proteins or peptides.

2. The activated branched biocompatible polymer derivatives according to claim 1, wherein the activated biocompatible polymers have one or more branched polymer structures.

3. The activated branched biocompatible polymer derivatives according to claim 1, wherein the activated branched biocompatible polymer derivatives are represented by following formula 1:

FORMULA 1

$(P\text{-}OCH_2CO\text{-}NH\text{-}CHR\text{-}CO\text{-})_n\text{-}L\text{-}Q_k\text{-}A$

Wherein,

P and Q is the same or different biocompatible polymer,

R is H or alkyl,

L is aliphatic linking moiety covalently linked to each P and Q,

A is activating functional group,

n is an integer between 2 and 3,

k is an integer between 0 and 1.

4. The activated branched biocompatible polymer derivatives according to claim 1, wherein the biocompatible polymer has 200~100,000 of the molecular weight.

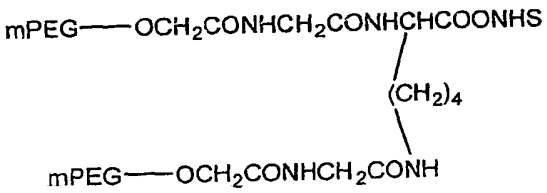
5. The activated branched biocompatible polymer derivatives according to claim 1, wherein the long length of polymer linker has 2,000~20,000 of the molecular weight.

6. The activated branched biocompatible polymer derivatives according to claim 1, wherein the biocompatible polymer is selected one from the group consisting of polyethylene glycol (PEG), polypropylene glycol (PPG), polyoxyethylene (POE), polytrimethylene glycol, polylactic acid and its derivatives, polyacrylic acid and their derivatives, polyamino acids, polyurethane, polyphosphazene, poly(L-lysine), polyalkylene oxide (PAO), water soluble polymers such as polysaccharide, dextran, and non-immunogenic polymers such as polyvinyl alcohol and polyacryl amide.

7. The activated branched biocompatible polymer derivatives according to claim 1, wherein the functional group of activated biocompatible polymer derivatives is selected one from the group consisting of -NHS, $-NHNH_2$, carbonyl imidazole, nitrophenyl, isocyanate, sulfonyl chloride, aldehyde, glyoxal, epoxide, carbonate, cyanuric halide, dithiocarbonate, tosylate and maleimide.

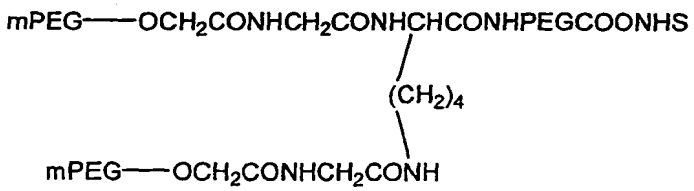
8. The activated branched biocompatible polymer derivatives according to claim 1, wherein the activated branched biocompatible polymer derivative is represented by formula 2:

FORMULA 2



9. The activated branched biocompatible polymer derivatives according to claim 1, wherein the activated branched biocompatible polymer derivative is represented by formula 3:

FORMULA 3



10. Protein-polymer or peptide-polymer conjugates, wherein the activated branched biocompatible polymer derivatives according to claim 1 react to the biologically active proteins or peptides.

11. The protein-polymer or peptide-polymer conjugates according to claim 10, wherein a molar ratio of the protein or peptide and the activated biocompatible polymer is 1:1 to 1:100 and preferably 1:1 to 1:20.

12. The protein-polymer or peptide-polymer conjugates according to claim 10, wherein the binding residue in protein or peptide is selected one from the group consisting of amino group, carboxyl group, carbonyl group, and oxidized sugar group.

13. The protein-polymer or peptide-polymer conjugates according to claim 10, wherein the protein or peptide is

selected one from the group consisting of alpha -, beta-, gamma- interferon, asparaginase, arginase, arginine deiminase, adenosine deaminase, superoxide dismutase, endotoxinase, catalase, chymotrypsin, lipase, uricase, adenosine diphosphatase, tyrosinase, glucose oxidase, glucosidase, galactosidase, glucouronidase, hemoglobin, blood factors (VII, VIII and IX), immunoglobulins, cytokines such as interleukins, G-CSF, GM-CSF, PDGF, lectins, ricins, TNF, TGFs, epidermal growth factor, human growth hormone, calcitonin, PTH, insulin, enkephalin, GHRP, LHRH and derivatives, calcitonin gene related peptide, thyroid stimulating hormone and thymic humoral factor.

Figures

1/5

FIGURE 1

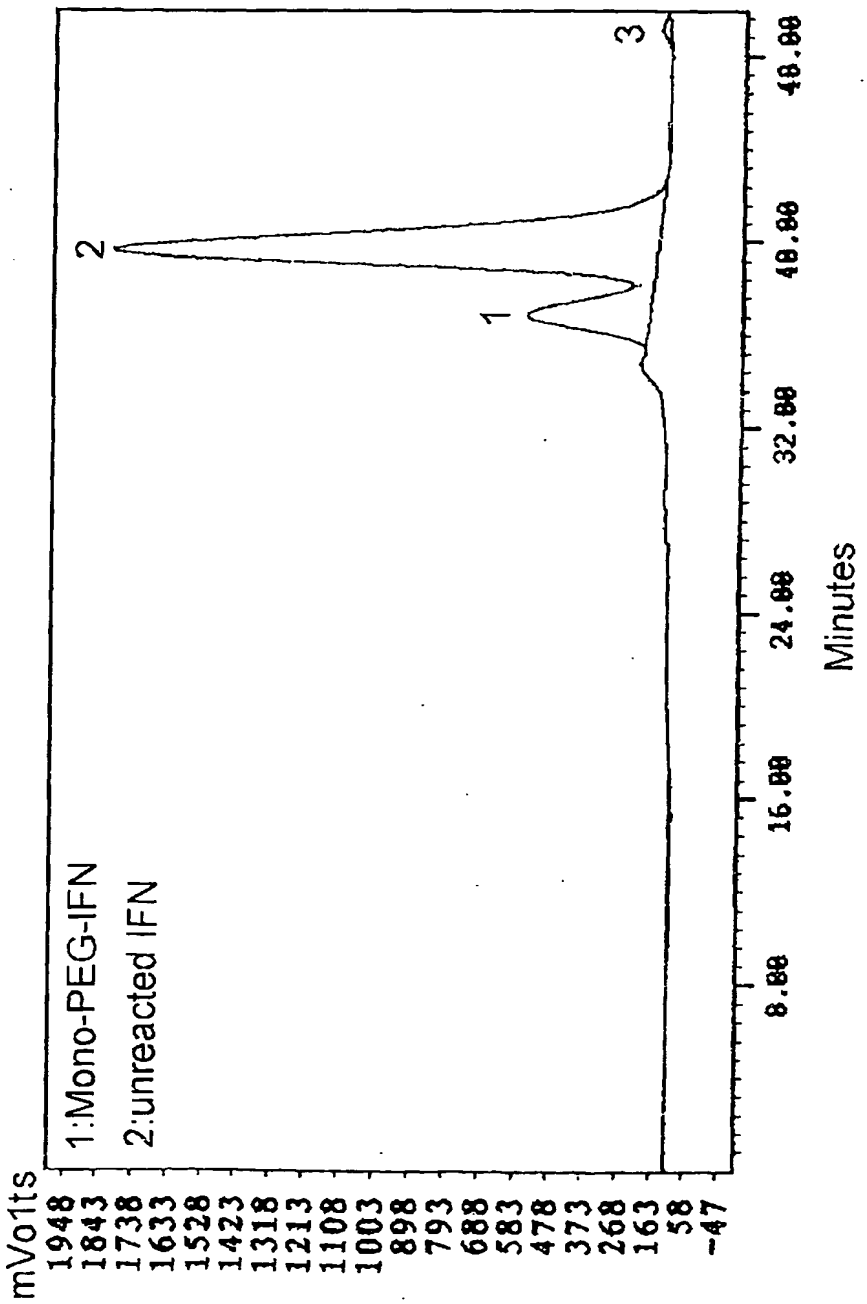
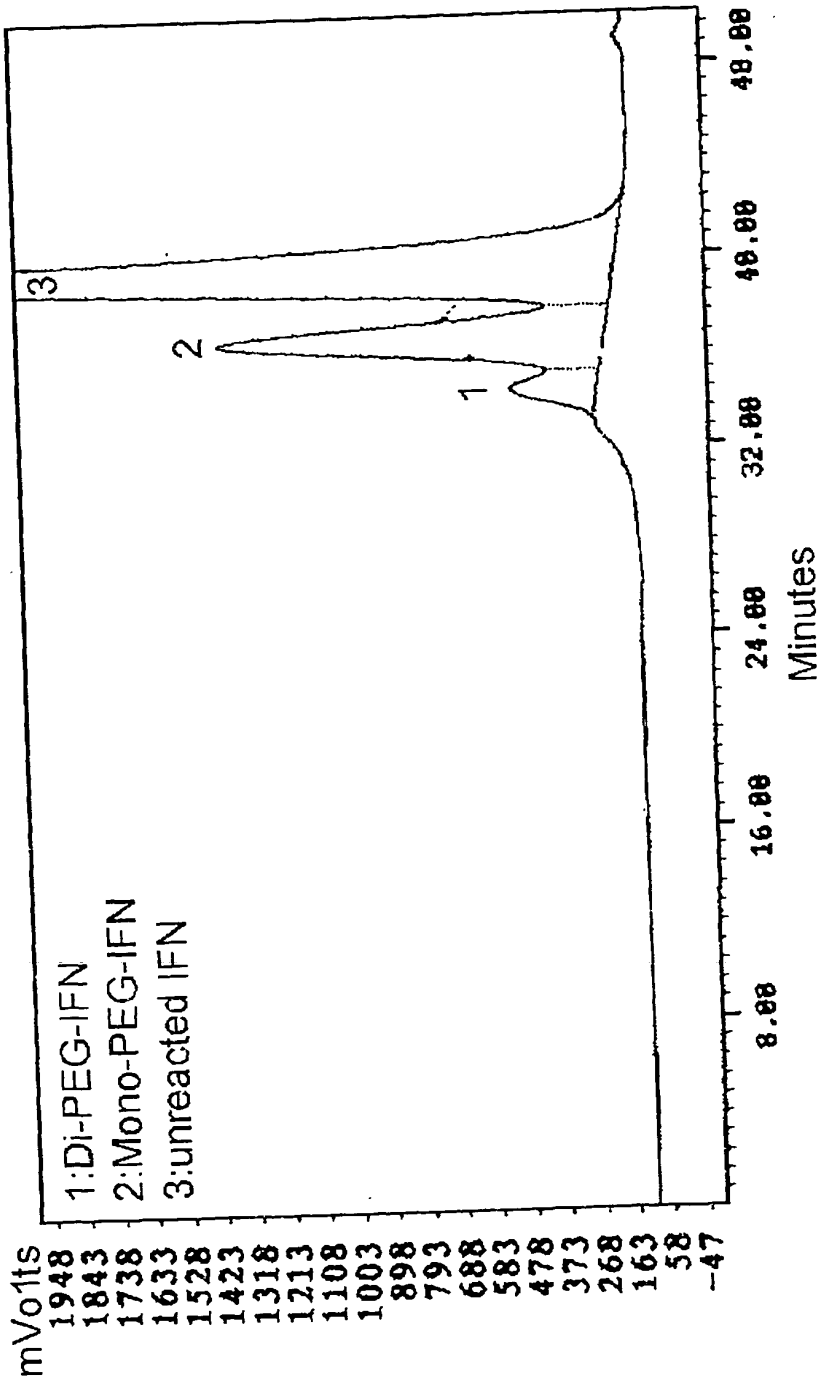


FIGURE 2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1003
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163
58
-47
1:Di-PEG-IFN
2:Mono-PEG-IFN
3:unreacted IFN
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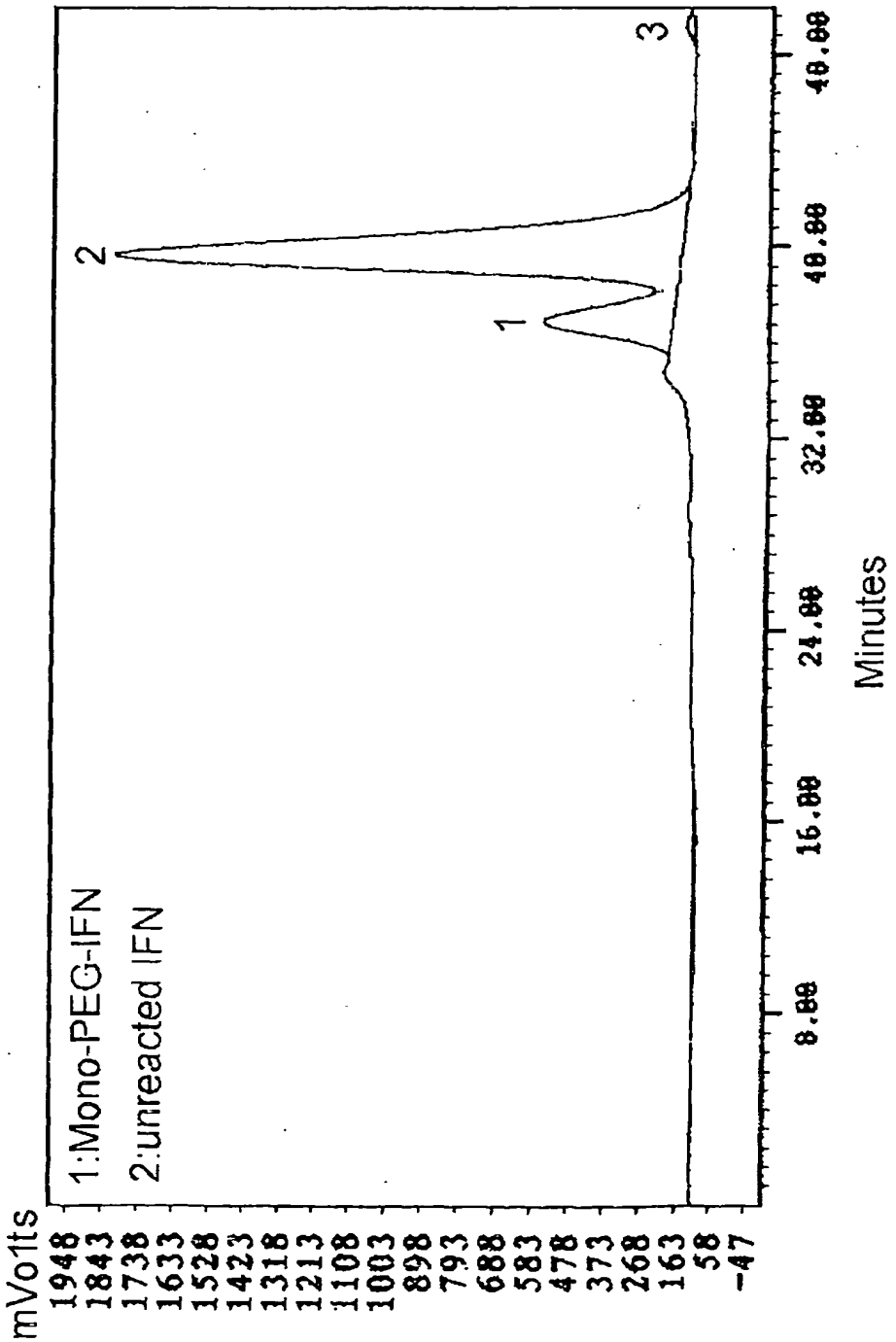
3/5

FIGURE 3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2:unreacted IFN
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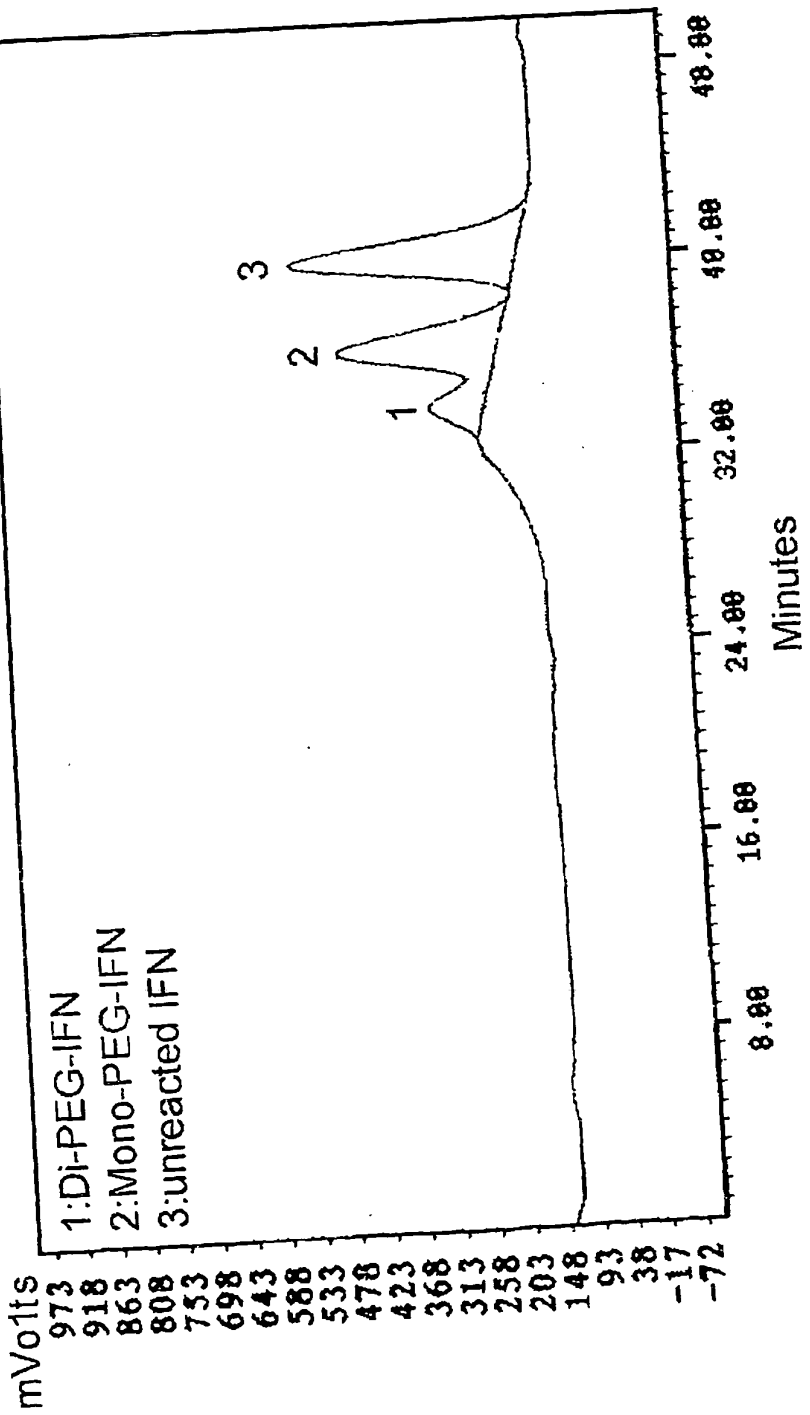
FIGURE 4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1:Di-PEG-IFN
2:Mono-PEG-IFN
3:unreacted IFN
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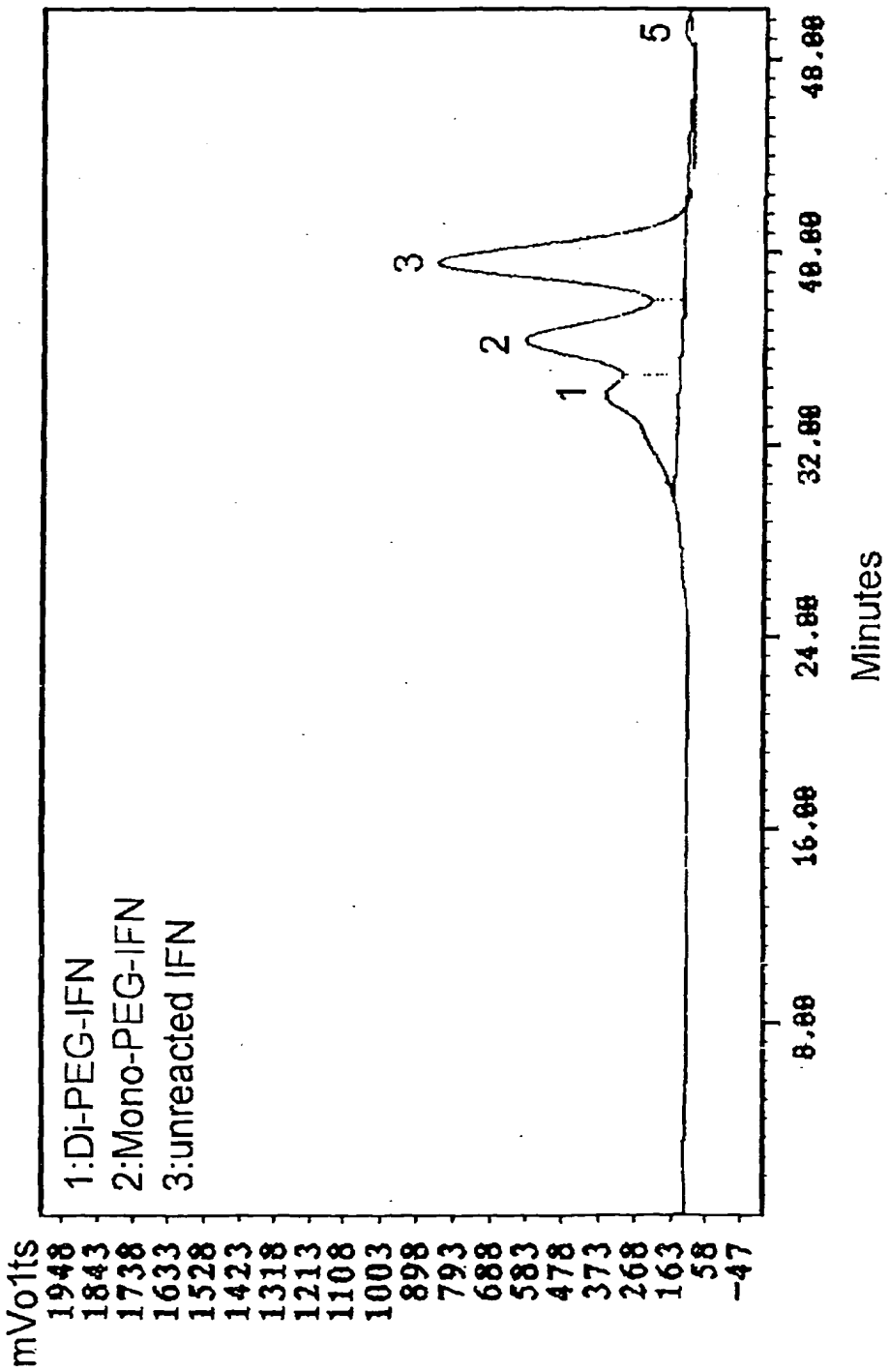
Minutes

FIGURE 5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3:unreacted IFN
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01209

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean Patents and applications for inventions since 1975
Korean Utility models and applications for Utility models since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

NPS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Lee et al., "Prolonged circulating lives of single-chain Fv Proteins conjugated with polyethylene glycol: A Comparison of conjugation chemistries and compounds" In Bioconjugate Chem. (1999), 10(6), pages 973-981.	1-7, 10-13
Y	Huang et al., "A polyethylene glycol copolymer for carrying and releasing multiple copies of cysteine-containing peptides" In Bioconjugate Chem. (1998), 9(5), pages 612-617.	1-7, 10-13
A	US 5854194 A (COLGATE-PALMOLIVE CO.) 29. December 1998 (29. 12. 1998) see entire document.	1-13
A	EP 1008355 A1 (DEBIO RECHERCHE PHARCEUTIQUE S.A.) 14. June 2000 (14. 06. 2000) see entire document.	1-13
A	WO 9958694 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 18. November 1999 (18. 11. 1999) see entire document.	1-13
A	Wang & Ikai, "Protein stretching III: force-extension curves of tethered bovine carbonic anhydrase B to the siliconsubstrate under native, intermediate and denaturing conditions" In Jpn. J. Appl. Phys., Part 1 (1999), 38(6B), pages 392-3917.	1-13

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 SEPTEMBER 2001 (27.09.2001)	28 SEPTEMBER 2001 (28.09.2001)
Name and mailing address of the ISA/KR Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer Yoon, Kyung Ae Telephone No. 82-42-481-5609

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01209

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5854194 A	29. 12. 1998	US 5955407 A US 6020301 A	21. 09. 1991 01. 02. 2000
EP 1008355 A1	14. 06. 2000	WO 2000033881A1	15. 06. 2000
WO 9958694 A1	18. 11. 1999	US 2610707 B1 AU 9939834 A1 EP 1078079 A1	03. 04. 2001 29. 11. 1999 28. 02. 2001

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
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(43) Internationales Veröffentlichungsdatum
28. März 2002 (28.03.2002)

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14. September 2001 (14.09.2001)

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(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
100 47 204.4 23. September 2000 (23.09.2000) DE

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(81) Bestimmungsstaaten *(national)*: AU, BG, BR, BY, CA, CN, CZ, DZ, HU, ID, IL, IN, JP, KR, MX, NO, NZ, PL, RO, RU, SG, SI, SK, UA, US, UZ, VN, YU, ZA.

(84) Bestimmungsstaaten *(regional)*: europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Veröffentlicht:
— *mit internationalem Recherchenbericht*
— *vor Ablauf der für Änderungen der Ansprüche geltenden Frist; Veröffentlichung wird wiederholt, falls Änderungen eintreffen*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 18. Juli 2002

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

WO 02/024156 A3

(54) Title: TOPICAL SKIN-TREATMENT AGENT CONTAINING ARGINASE

(54) Bezeichnung: TOPISCHE HAUTBEHANDLUNGSMITTEL MIT ARGINASE

(57) Abstract: The invention relates to the use of arginase as a component in cosmetic skincare preparations for improving the condition of the skin and for producing dermatological preparations for the topical treatment of the skin. Said cosmetic preparations preferably contain between 0.001 and 1000 units (U) of the arginase enzyme per 100 g of the preparation and protect the skin against dryness, roughness and inflammation.

(57) Zusammenfassung: Die Erfindung betrifft die Verwendung von Arginase als Komponente zur Verbesserung des Hautzustandes in kosmetischen Zubereitungen zur Pflege der Haut und zur Herstellung dermatologischer Zubereitungen zur topischen Behandlung der Haut. Kosmetische Zubereitungen enthalten bevorzugt 0,001 bis 1000 Einheiten (U) des Enzyms Arginase pro 100 g der Zubereitung und dienen dem Schutz der Haut gegen Trockenheit, Rauhigkeit und Entzündlichkeit.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10617

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K7/48 A61K38/43

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, BIOSIS, EPO-Internal, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 419 901 A (GRIFFITH) 30 May 1995 (1995-05-30) cited in the application the whole document ---	1-4
A	WO 97 15280 A (L'OREAL) 1 May 1997 (1997-05-01) cited in the application the whole document ---	1-4
A	DE 198 16 072 A (WELLA) 14 October 1999 (1999-10-14) the whole document ---	1-4
A	EP 0 956 864 A (KYOWA HAKKO KOGYO CO.) 17 November 1999 (1999-11-17) the whole document --- -/--	1-4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 May 2002	13/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fischer, J.P.

1

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/10617

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 01 64858 A (MILLENNIUM PHARMACEUTICALS) 7 September 2001 (2001-09-07) the whole document	1-4
A	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 05 (C & JP 10 007581 A (ADVANCED SUKIN RES KENKYUSHO KK) cited in the application abstract	1-4

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In[illegible]ational Application No
PCT/EP 01/10617

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5419901	A	30-05-1995	US	5196195 A	23-03-1993
			US	5395612 A	07-03-1995
WO 9715280	A	01-05-1997	FR	2740339 A1	30-04-1997
			CA	2222303 A1	01-05-1997
			EP	0859591 A1	26-08-1998
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			JP	10511404 T	04-11-1998
			JP	3110050 B2	20-11-2000
			NO	976142 A	03-06-1998
			US	2002037854 A1	28-03-2002
DE 19816072	A	14-10-1999	DE	19816072 A1	14-10-1999
EP 956864	A	17-11-1999	AU	5192498 A	29-06-1998
			EP	0956864 A1	17-11-1999
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			WO	0164858 A2	07-09-2001
JP 10007581	A	13-01-1998	NONE		

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP 01/10617

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 A61K7/48 A61K38/43

Nach der internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 7 A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)
WPI Data, PAJ, BIOSIS, EPO-Internal, CHEM ABS Data, EMBASE

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	US 5 419 901 A (GRIFFITH) 30. Mai 1995 (1995-05-30) in der Anmeldung erwähnt das ganze Dokument ---	1-4
A	WO 97 15280 A (L'OREAL) 1. Mai 1997 (1997-05-01) in der Anmeldung erwähnt das ganze Dokument ---	1-4
A	DE 198 16 072 A (WELLA) 14. Oktober 1999 (1999-10-14) das ganze Dokument ---	1-4
A	EP 0 956 864 A (KYOWA HAKKO KOGYO CO.) 17. November 1999 (1999-11-17) das ganze Dokument --- -/--	1-4

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

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"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
6. Mai 2002	13/05/2002

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax (+31–70) 340–3016	Fischer, J.P.

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP 01/10617

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A,P	WO 01 64858 A (MILLENNIUM PHARMACEUTICALS) 7. September 2001 (2001-09-07) das ganze Dokument	1-4
A	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 05 (C & JP 10 007581 A (ADVANCED SUKIN RES KENKYUSHO KK) in der Anmeldung erwähnt Zusammenfassung	1-4

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INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlic. .gen, die zur selben Patentfamilie gehören

Ir ationales Aktenzeichen
PCT/EP 01/10617

Im Recherchenbericht angeführtes Patentdokument		Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
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			US	5395612 A	07-03-1995
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			CA	2222303 A1	01-05-1997
			EP	0859591 A1	26-08-1998
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			JP	3110050 B2	20-11-2000
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JP 10007581	A	13-01-1998	KEINE		